Studies on Experimental Enteric Salmonellosis in Ponies

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ABSTRACT

Clinical, bacteriological, serological and haematological observations were made on 13 adult ponies orally inoculated with Salmonella typhimurium. The results were compared to two control ponies and four others infected by accidental transmission. The clinical responses in inoculated ponies included pyrexia lasting four days and neutropaenia during the first five days after inoculation followed by a neutrophilia. Pyrexia and neutropaenia was associated with maximal shedding of organisms in the rectal faeces. Changes in the character of the faeces occurred between one and two days after inoculation and appeared to be associated with the serological response. Serological responses occurred in all the infected ponies except one. At necropsy, of the 14 ponies with positive cultures in the colon, seven had negative cultures in the rectal faeces. Serological studies performed on 43 clinically normal horses indicated a correlation between age and salmonella agglutination titre.

RÉSUMÉ

Cette expérience visait à obtenir des renseignements cliniques, bactériologiques, sérologiques et hématologiques, chez 13 poneys adultes soumis à une infection buccale expérimentale avec Salmonella typhimurium. On compara ensuite les résultats avec ceux qu'on obtint chez deux poneys témoins et chez quatre autres, victimes d'une contamination accidentelle. L'infection expérimentale provoqua une hyperthermie qui dura quatre jours, ainsi qu'une neutropénie qui persista durant cinq jours; on décela ensuite une neutrophilie. L'hyperthermie et la neutropénie coïncidèrent avec l'élimination fécale maximale de salmonelles. Le ramollissement des fèces se produisit de un à deux jours après l'infection et sembla coïncider avec l'apparition des anticorps sériques que l'on décela chez 12 des 13 poneys expérimentaux. Lors de la nécropsie, on nota que des 14 poneys dont le côlon recelait des salmonelles, sept n'en éliminaient pas dans leurs fèces. Une étude sérologique, qui portait sur 43 chevaux apparemment normaux, révéla une corrélation entre l'âge et le taux d'anticorps agglutinants à l'endroit des salmonelles.

INTRODUCTION

Salmonellosis in equidae is a widely recognized disease and while a number of clinical cases have been reported (9, 14), there has been very little experimental work published (3, 7).

The purpose of this paper is to report observations on ponies that were experimentally infected with *Salmonella typhimurium*.

MATERIALS AND METHODS

Nineteen ponies of varying age and sex weighing between 150 and 250 kg were purchased from a stockyard. All ponies underwent a preliminary clinical examination. Their ages were determined by dental examination.

Initially, one pony was selected for a pilot study to ensure that infection by salmonella could be achieved and to establish the general course of the disease. Subsequently, 12 ponies were separated into three groups of four for infection and the remainder were separated into three groups

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of two for controls. The pilot pony and each of the six groups were housed separately in isolation units which had double sealed doors with foot bath and a shower between the doors. The units were entered twice daily by the grooms for feeding and cleaning and once daily by the technicians for examination and sampling of the ponies. After leaving each unit, personnel either showered, if wearing water proof clothing, or changed protective clothing. Duties in the noninfective units were completed before entering the infected ones. The ponies received a pelleted horse ration twice daily, had free access to water and were free to wander as a group around their respective isolation units.

During the 18 days of this study the ponies were monitored almost daily as follows:

(a) Clinical assessment of each pony was made by recording rectal temperature, pulse and respiratory rates. The character of the faeces was recorded as being normal, mild diarrhoea, watery diarrhoea or dysentery.

(b) Blood samples for haematological examination were drawn from the jugular vein into an E.D.T.A. vacuum tube. Total red and white cell counts, packed cell volume and haemoglobin determinations were made using a Model S Coulter counter. White blood cell differential counts were made for 100 cells from Wright's stained blood smears.

(c) Bacteriological studies were performed on faecal samples collected from the rectum of each pony. A two gram portion was inoculated into 20 ml of tetrathionate broth, incubated at 37°C and subcultured at 24 and 48 hrs onto MacConkey/Nalidixic acid plates. In addition, tenfold dilutions of the faeces were made in physiological saline and from each dilution, measured volumes were surface inoculated onto MacConkey/Nalidixic acid plates. After 48 hrs incubation the Nalidixic acid resistant colonies were counted and the viable count determined.

(d) Serological studies were performed on blood drawn from the jugular vein. The antigen for the serological test was prepared from the stock cultures of *S. typhimurium* (472) by inoculating trypticase soy broth (TSB) and incubating for 18 hours on a shaker in 37°C water bath. The organisms were washed three times in sterile physiological saline solution (pH 7.2). The final pellet was suspended in 0.5% formalinized saline and made up to a cell concentration comparable with a McFarland Nephelometer #2 tube reading. Beginning with a 1/10 dilution of serum in physiologic saline, serial twofold dilutions were prepared in 0.5 ml amounts. To each tube in the series 0.5 ml of antigen suspension was added. After 24 hour incubation at 37°C the tubes were examined for agglutination. Agglutination titres for *S. typhimurium* were also performed on the sera obtained from 43 horses clinically free from signs of salmonellosis and varying in age from three months to 20 years.

During the first three days, the ponies were allowed to acclimatize, then on the fourth and fifth days, the designated animals were inoculated with *S. typhimurium* (strain 472). This strain had been isolated from a horse dying of salmonellosis and was subsequently made resistant to Nalidixic acid for use in the study. The organism was passed through mice and upon reisolation was grown in TSB and lyophilized to serve as the stock culture. The infective inoculum was prepared by seeding a stock culture in TSB and incubating it in a shaking water bath for 48 hours at 37°C. Viable cell counts were made by surface inoculation of MacConkey/Nalidixic acid (30 μg/ml) plates prior to inoculation. The ponies received 10^9 organisms of *S. typhimurium* in a ten ml volume administered by stomach tube.

After the 18 days observations reported in this study, the ponies were maintained until day 35 for other purposes. On the 35th day, faecal samples were collected from the rectum of each pony, then the ponies were killed with intravenous barbiturate and ingesta was obtained immediately from the pelvic flexure. Sections of bowel at the jejuno-ileal junction and the pelvic flexure were obtained together with sections of spleen, liver and caecal lymph node. The samples obtained on day 35 were cultured for the presence of *S. typhimurium* by the same methods as described for faeces.

RESULTS

Salmonellae were not isolated from any of the ponies prior to inoculation. Two of the three groups of nonicoculated ponies became infected with *S. typhimurium* strain 472 naR soon after the beginning of

the experiment. This was due to the accidental transfer of the organism by personnel moving from infected to control groups without the prescribed precautions.

INOCULATED PONIES

As the main sign of enteric salmonellosis is diarrhoea, the responses of the 13 inoculated ponies were categorized according to the character of the faeces before and after inoculation.

Type I. Normal prior to inoculation but with mild diarrhoea occurring from six to 11 days after inoculation (Fig. 1).

Six ponies showed this type of response. They were mainly young, four were two year olds. They had the lowest preinoculation titres and their response appeared to be primary as there was a five to seven day delay before a rise in titre was noted. All six had an elevation in rectal temperature for four days after inoculation but there was no significant rise in pulse and respiratory rates. They showed a marked leukopaenia and neutropaenia within 24 hours following inoculation which persisted up to five days. Subsequently, there was a leukocytosis and neutrophilia which was still present 14 days after inoculation. The numbers of organism excreted daily in the faeces reached a peak within three days following inoculation and then decreased.

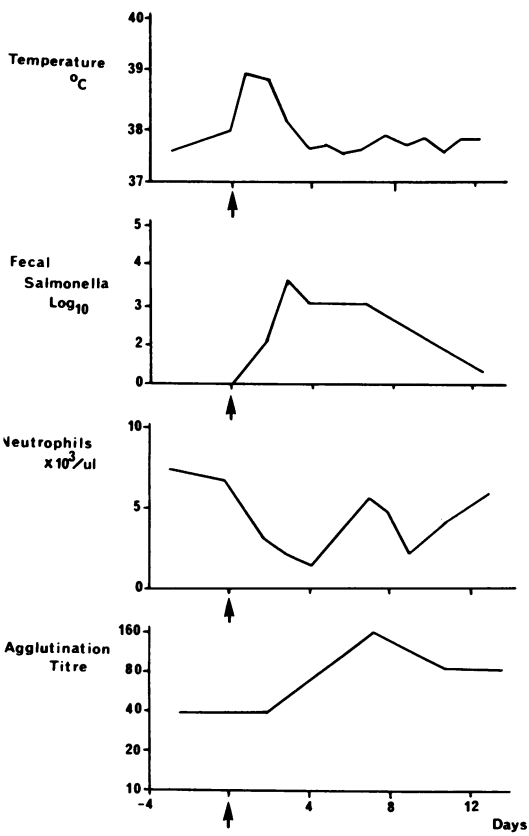

Fig. 1 The temperature, faecal Salmonella counts, neutrophil numbers and agglutination titres of six normal ponies infected orally with 10^9 S. typhimurium.

Type II. Normal faeces throughout the experiment (Fig. 2).

These two ponies were older than 15 years of age. They had a higher preinoculation titre than type I ponies and showed a typical anamnestic response as the rise in titre occurred immediately after the challenge, indicating previous exposure to salmonella. All other clinical observations and laboratory results resembled the type I response.

Type III. Initial diarrhoea with a watery diarrhoea three to seven days after inoculation (Fig. 3).

The four ponies which exhibited this response varied in age. Two were two year olds, one was 11 years and one was 15 years old. These ponies were diarrhoeic and pyretic prior to inoculation, suggesting they had an enteric infection at the start of the experiment. The mean serological titre of this group prior to inoculation was higher than type I. Serologically, the latent infection in these type III ponies appeared to be due to salmonellosis.

Starting three days after inoculation these ponies had a rise in titre which reached a higher peak than the type I response and so appeared to be an anamnestic response. Even though these ponies were initially pyretic they also had a rise in rectal temperature for four days following inoculation. All other clinical observations and laboratory results resembled the type I response.

Type IV. Diarrhoea before inoculation followed by profuse watery diarrhoea within 24 hours followed by dysentery on day 15 (Fig. 4).

Prior to inoculation, this two year old pony was pyretic, diarrhoeic and had the highest preinoculation titre. These are characteristics typical of enteric salmonel-

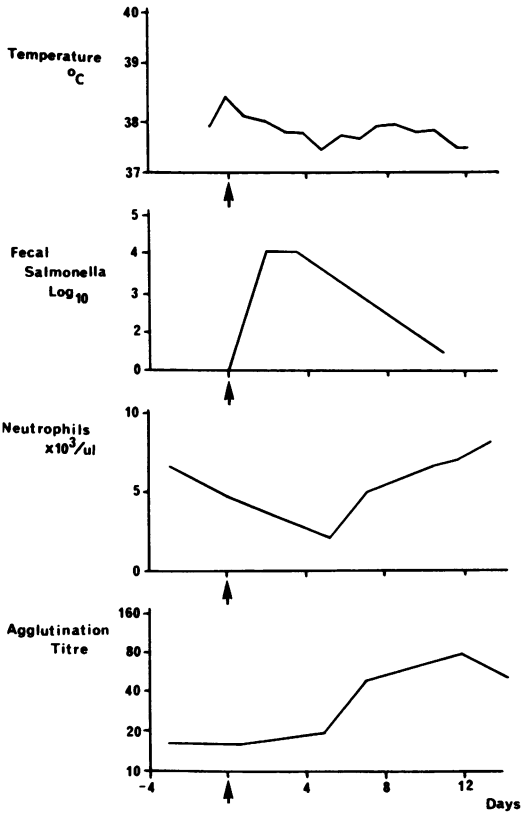

Fig. 2. The temperature, faecal Salmonella counts, neutrophil numbers and agglutination titres of two normal ponies infected orally with 10^9 S. typhimurium.

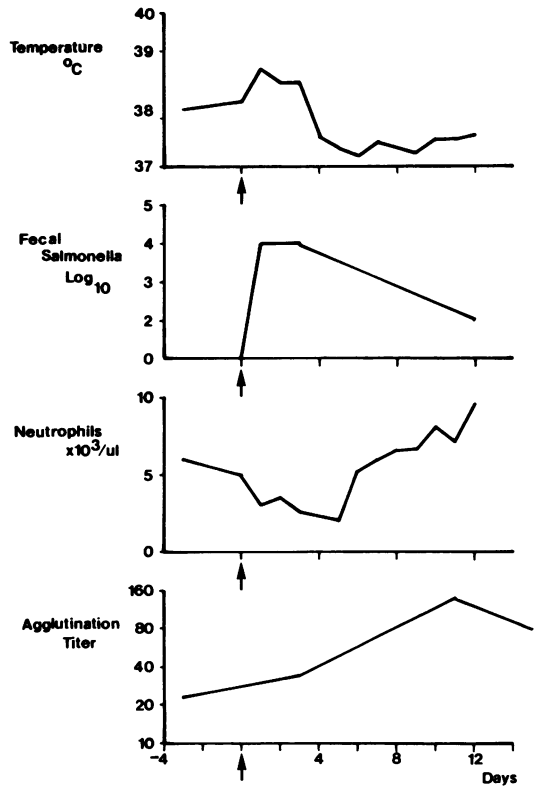

Fig. 3. The temperature, faecal Salmonella counts, neutrophil numbers and agglutination titres of four diarrhoeic ponies infected orally with 10^9 S. typhimurium.

losis but this animal was not excreting salmonella.

Unlike the type III response, this pony deteriorated in condition after inoculation. Its serological response was immediate and prolonged. Similarly, its rectal temperature remained elevated. Its preinoculation pulse rate of 60/minute was as high as the other ponies reached after inoculation. After inoculation, this pony's pulse rate rose to 72/minute. There was no significant alteration in respiratory rate. The white cell response resembled the other types except in addition it had a continuous lymphopaenia. The other haematological results demonstrated a significant degree of haemoconcentration following inoculation (Fig. 5). The numbers of organisms excreted daily in the faeces differed significantly from the other types as organisms continued to be excreted at the high rate of 10^5 organisms/gm up to and including day 18.

Accidentally Infected Ponies

Four ponies became infected and two aged animals of the group developed diarrhoea. Their serological titres were 1/20 and 1/40 but only one pony had a serological response after infection. A significant difference from the inoculated ponies was that diarrhoea occurred at the same time as the excretion of organisms commenced. There was no pyrexia, alteration in pulse and respiratory rates and no significant leukopaenia or neutropaenia.

Control Ponies Free of Salmonella Infection (Fig. 6)

Both ponies, one two year old and one four year old, had normal faeces throughout. Their agglutination titres were 1/40 which remained unchanged. Haematology results showed that initially they had a mild leukocytosis and neutrophilia followed by mild fluctuations thereafter. All other

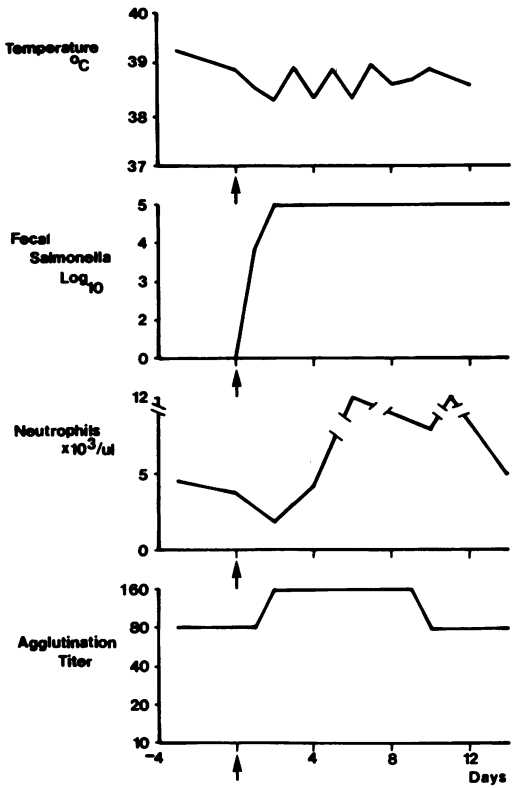

Fig. 4. The temperature, faecal Salmonella counts, neutrophil numbers and agglutination titres of a single pony severely affected with colitis and infected orally with 10^9 S. typhimurium.

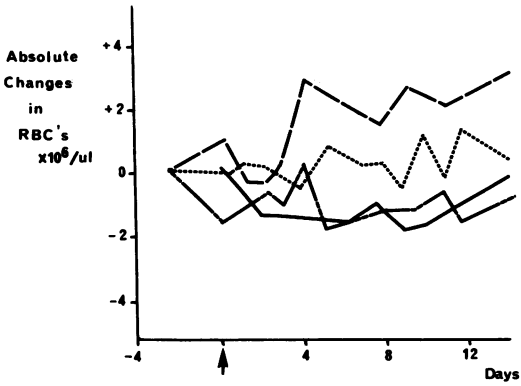

Fig. 5. Absolute changes in red blood cell counts in red blood cell counts in ponies infected orally with 10^9 S. typhimurium.
(————) noninfected — two ponies
(- - - - - - - -) experimentally infected — 12 ponies.
(— — — —) accidentally infected — four ponies
(— - - — - -) acute colitis — one pony

clinical observations and laboratory results remained constant.

Terminal Bacteriological Studies

The sites of salmonella isolation at necropsy are given in Table I.

Serological Studies

The serum agglutination titres of 43 clinically normal horses varied between 0-1/128. In the population examined there was a direct relationship between titre and the horse's age (R = 0.767 P 0.01) (Fig. 7). None of the seronegative horses was older than two years and the majority were foals less than one year old.

The mean serum agglutination titres of the 19 ponies prior to inoculation was within the age/titre range in Fig. 7 except for the pony which developed dysentery (Table IV). The preinoculation base-line titre was established on two or more tests and showed good reproducibility. Thus, one could detect with some assurance the changes which occurred. The peak titres occurred six to 12 days postinoculation with the exception of pony 43 in which the response was immediate.

DISCUSSION

Pathogenic strains of salmonella have been shown to cause diarrhoea by invading and damaging the gut wall, resulting in fluid secretion (6). It was anticipated that diarrhoea would occur in the heavily inoculated ponies within 24 to 84 hrs as has been reported to occur in foals (3). Except for the type IV pony, the daily numbers of salmonellae per gram of faeces in all ponies were similar (Fig. 1-3) and yet two ponies did not have diarrhoea and the other two ponies had diarrhoea or watery diarrhoea beginning at varying intervals between three and 11 days after inoculation. Even though culturing rectal faeces cannot accurately represent the bacterial population in the colon, it must reflect the trends. The change in the character of the faeces did not appear to be associated with the numbers of organisms in the faeces.

Only in two accidentally infected controls did the initial detection of salmonella coincide with the start of diarrhoea. The discrepancy between the onset of diarroea and positive culture in the intentionally and the accidentally inoculated ponies can

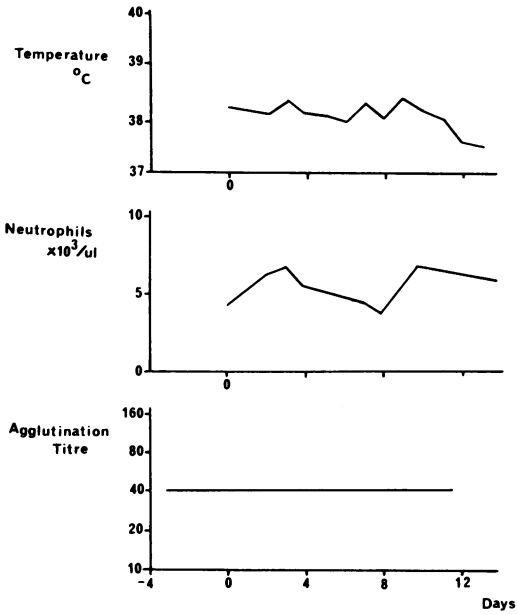

Fig. 6. The temperature, neutrophil numbers and agglutination titres of two noninfected control ponies.

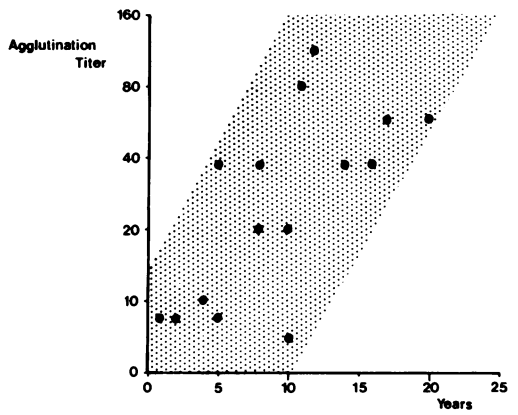

Fig. 7. The serum agglutination titres of 43 clinically normal horses in relation to their age.

only be explained when the pathogenesis of equine salmonellosis is more fully understood.

Diarrhoea in some bacterial enteric disease such as *E. coli* is the result of enterotoxin production (13). Previously it was thought that salmonella did not produce enterotoxin although some some recent investigations (8, 12) do indicate this possibility. The one factor that seemed to coincide with the change in character of the faeces in the experimentally inoculated ponies was the serological response.

The ponies grouped as a type I reaction developed diarrhoea six to 11 days after inoculation and showed the most significant rise in titre five to seven days after inoculation (Fig. 1). Similarly, the ponies of type III developed a watery diarrhoea three to five days after inoculation and showed a rise in titre starting three days after inoculation (Fig. 3). The pony with type IV reaction developed a watery diarrhoea and showed an anamnestic response within 24 hours of inoculation (Fig. 4).

It has generally been accepted that serological examination of horses suspected of having salmonellosis is of limited value because of the high background level of antibodies and secondly because titre elevation may not be marked (1, 2). Two factors may account for this undramatic response. First, salmonella infection also stimulates a cell mediated immunological response, so an agglutination test is not truly assessing the immune status of the animal (11). Secondly, the epidemiological features of salmonella infections are complex (9). In a study of the serology of equine salmonellosis, Baker (1) indicated that a titre of 1:240 was required to confirm that a horse had been infected by salmonellae. This conclusion was based on the observation that clinically normal horses could have titres of up to 1:160.

In the present study, none of the inoculated and accidentally infected ponies developed titres greater than 1/160 (Figs. 1-4) even though an homologous antigen was used and all ponies were clinically affected. All but one of the inoculated ponies showed a serological response and the exception had one of the highest preinoculation titres. In this study a titre in excess of 1/240 was not necessary to indicate exposure to the organism.

A statistical significant correlation between age and titre was noted in the 43 clinically normal horses (Fig. 7). As salmonella is such an ubiquitous organism, this trend of a rise in titre with age was probably due to greater opportunity for exposure to salmonella. The serological status of the ponies used in this experiment prior to infection were, except for one pony, in the same range as the clinically normal horses (Fig. 7). This indicates they were a representative population of ponies.

As serological responses occur when horses show clinical signs of salmonellosis,

TABLE I. Isolation of *S. typhimurium* from Experimental Ponies at Necropsy

Clinical Response	Pony No.	Rectal Faeces	Pelvic Flexure Contents	Colonic Wall	Ileal Wall	Liver	Spleen	Caecal Lymph Node
Type I	1	−	+	+	+	−	−	+
	2	−	−	−	+	−	−	−
	3	−	−	−	−	−	−	−
	5	−	+	+	−	−	−	−
	8	+	+	+	+	−	−	−
	13	−	+	−	−	−	−	−
Type II	7	−	+	+	−	−	−	−
	10	−	−	−	−	−	−	−
Type III	6	+	+	+	−	−	−	+
	11	+	+	+	−	−	−	−
	15	−	+	−	−	−	−	−
	16	+	+	+	−	−	−	−
Type IV	4	+	+	+	+	+	+	+
Controls	9	+	+	+	+	−	−	−
(Accidentally infected)	12	+	+	+	−	−	−	−
	14	−	+	+	+	−	−	−
	17	−	+	+	−	−	−	−
Controls	18	−	−	−	−	−	−	−
(noninfected)	19	−	−	−	−	−	−	−

paired sera samples revealing a rise in titre would therefore be a valuable diagnostic aid. A single titre may not be of any value except in a foal which has a positive titre or in a young horse that has an exceptionally high titre outside the normal range discussed in this study.

Unlike the change in character of the faeces and the serological response, neutropaenia and pyrexia do appear to be related to the number of organisms in the rectal faeces. This implicates the production and excessive absorption of endotoxin during the four days after infection (4, 10, 15). All 12 of the inoculated ponies, type I, II and III showed a neutropaenia below 3,600/μl with a relative percentage drop after infection on day 4 of between 27 and 34% (Figs. 1-3). The dysenteric pony (Type IV) had a neutropaenia of 1700/μl and the relative drop was 49% (Fig. 4). Dorn *et al* (5) reported that in eight out of nine horses which developed pyrexia and diarrhoea with positive salmonella cultures, a neutropaenia (3,600 neutrophils/μl) or a decline greater than 14% in total neutrophils occurred. The authors considered this degree of neutropaenia to be specific for salmonellosis.

The two control ponies that remained free of salmonella infection, showed a drop in total white cell and neutrophil counts after day 7. As the neutrophil counts in both ponies reached 3,600/μl and the percentage drop varied between 22 and 42%, the percentage drop as discussed by Dorn (5) cannot be considered specific for salmonellosis.

The two noninfected ponies epitomize the problem for the clinician in a surgical clinic. A series of previous values are usually not available for comparison so that if the white cell count is high at admission due to physiological response of endogenous steroid and subsequently drops because the horse happens to have a low normal count, interpretation of this neutropaenia as an indication of salmonellosis could be possible. This physiological response appears to have happened in these noninfected ponies. They were the only group of ponies to show an initial leukocytosis which was probably physiological. A concurrent lymphopaenia supports this hypothesis. Even though the inoculated ponies demonstrated a classical neutropaenia, the accidentally infected ponies did not. Although there was a gradual drop in the white cell count to a low of 3,500/μl, it was not dramatic and so a significant neutropaenia does not necessarily occur following salmonella infection in horses.

The first sign of enteric salmonellosis to the clinician, other than pyrexia, is usually diarrhoea. As the neutropaenia occurs within 24 hours of infection and diarrhoea may

not develop up to six days later, a neutropaenia may or may not be detected. Lymphocyte counts showed mild fluctuations in all the ponies but in the type IV pony with dysentery, the total count was low and continued to drop even though a significant neutrophilia occurred after day 9. The reason for the lymphocyte response is not clear.

Packed cell volume, haemoglobin and erythrocyte counts did not change following inoculation except in the pony with dysentery (Fig. 5). Changes in these haematological values in equine salmonellosis indicate a grave prognosis.

The cultures at postmortem confirm that a negative rectal swab does not indicate freedom from salmonella and is of limited significance as seven out of the 14 salmonella infected ponies with positive colonic cultures had negative rectal faecal cultures (Table I).

REFERENCES

1. **BAKER, J. R.** Salmonellosis in the horse. Br. vet. J. **126**: 100-105. 1970.
2. **BLOOD, D. C. and J. A. HENDERSON.** Veterinary Medicine — 4th Edition. pp. 335-362. London: Baillière Tindall. 1974.
3. **BRYANS, J. T., B. O. MOORE and M. W. CROWE.** Safety and efficacy of furozone in the treatment of equine salmonellosis. Vet. Med. small Anim. Clin. **60**: 626-633. 1965.
4. **BURROWS, G. E. Escherichia coli** endotoxemia in the conscious pony. Am. J. vet. Res. **32**: 243-248. 1971.
5. **DORN, C. R., J. R. COFFMAN, D. A. SCHMIDT, H. E. GARNER, J. B. ADDISON and E. J. McCUNE.** Neutropenia and salmonellosis in hospitalized horses. J. Am. vet. med. Ass. **166**: 65-67. 1975.
6. **GIANNELLA, R. A.** Pathogenesis of salmonella enteritis and diarrhea. In Microbiology. D. Schlessinger, Ed. pp. 170-173. Washington: American Society for Microbiology. 1975.
7. **GRAHAM, R., F. H. K. REYNOLDS and J. F. HILL.** Bacteriologic studies of a peracute disease of horses and mules. J. Am. vet. med. Ass. **56**: 378-393; 489-507 and 586-599. 1920.
8. **KOUPAL, L. R. and R. M. DEIBEL.** Assay, characterization and localization of an enterotoxin produced by Salmonella. Infection & Immunity **11**: 14-22. 1975.
9. **MORSE, E. V., M. A. DUNCAN, E. A. PAGE and J. F. FESSLER.** Salmonellosis in equidae. A study of 23 cases. Cornell Vet. **66**: 198-213. 1976.
10. **MULHOLLAND, J. M. and L. E. CLUFF.** The effect of endotoxin upon susceptibility to infection: The role of the granulocyte. In Bacterial Endotoxins. pp. 211-229. Ed. by M. Candy and W. Braun, Institute of Microbiology Rutgers, The State University, New Jersey. 1964.
11. **ROWLEY, D., I. AUZINS and C. R. JENKIN.** Further studies regarding the question of cellular immunity in mouse typhoid. Aust. J. exp. Biol. med. Sci. **46**: 447-463. 1968.
12. **SANDEFUR, P. D. and J. W. PETERSON.** Isolation of skin permeability factors from culture filtrates of **Salmonella typhimurium.** Infection & Immunity **14**: 671-679. 1976.
13. **STEVENS, J. B., C. L. GYLES and D. A. BARNUM.** Production of diarrhea in pigs in response to **Escherichia coli** enterotoxin. Am. J. vet. Res. **33**: 2511-2526. 1972.
14. **STUART, B. P., B. R. MARTIN, L. P. WILLIAMS, Jr. and H. VON BYEM.** Salmonella induced meningoencephalitis in a foal. J. Am. vet. med. Ass. **162**: 211-213. 1973.
15. **THOMAS, L.** The physiological disturbances produced by endotoxins. Ann. Rev. Physiol. **16**: 467-490. 1954.